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HANS PHILIPP POPPER

M.D., University of Vienna, 1928
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DISTRIBUTION OF VITAMIN A IN TISSUE AS VISUALIZED BY FLUORESCENCE MICROSCOPY*

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Attempts to localize biologically active substances in tissues by histo-chemical and histo-physical methods have been carried out repeatedly in order to clarify their rôle in the organism (1, 2). The demonstration of vitamin A by a histologic method seems to be important in view of its still problematic rôle in the body.

Vitamin A lends itself to morphologic demonstration because its fat solubility prevents its solution in aqueous fixatives. There have been some attempts to visualize it by histo-chemical methods, but these were of questionable specificity (3, 4, 5). In recent years the fluoresence of vitamin A in ultra-violet light has been used for its demonstration in tissues. This review presents the findings obtained by this method.

HISTORICAL INTRODUCTION. In 1932, Von Querner (6) described a luminescent substance (Leuchtstoff X) in isotropic fat droplets in the liver cells. Its strong fluorescence was quickly destroyed by the ultra-violet light eliciting this phenomenon. In further studies he (7) found this substance soluble in lipoid solvents and not in aqueous solutions. After various attempts to identify it by histo-chemical examinations, he (8) finally concluded that it is related to pigments, probably the fat soluble carotenoids. Since Querner encountered a similar fluorescence in fish liver oils and in vitamin A concentrates, he assumed that the luminescent substance found in liver, retina, adrenal cortex and pituitary body is vitamin A.

Querner deserves the credit for having pointed to the vitamin A fluorescence as a means of demonstrating vitamin A in tissues even though his histological descriptions were incorrect and the few animal experiments reported were insufficiently controlled. Prior to Querner, Hirt (9) described bright yellow fluorescent droplets in the wall of the liver sinusoids in living animals which he (10) later related to vitamin A. Querner localized the fluorescence in the lipoid droplets of the epithelial liver cells, whereas Hirt and his co-workers found them in the Kupffer cells. In a more recent, chiefly vital, microscopic study Hirt and Wimmer (11) found the greatest amount of vitamin A fluorescence in the Kupffer cells of the liver and endothelial cells of the lung, less in the endothelial cells of other organs. The parenchymatous cells were reported as free of vitamin A fluorescence. In addition, they found in living organs different fluorescent substances which they associated with various members of the vitamin B complex and different combinations of them with proteins, with vitamin C, or with the active liver principle of pernicious anemia. Their reports, so far accessible, do not give sufficient information as to how these different fluorescent details in the living organs may be differentiated and fail to offer con-

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trolled studies as to the specificity of the fluorescent phenomena. As long as there is not more known about the basis for their assumption, Ellinger's (12) criticism against their theories appears justified. Nevertheless, their observations in living organs are important for the evaluation of the somewhat clearer pictures of fixed specimens. Far better substantiated appear the observations of Jancsó and Jancsó (13) who observed an increase of the characteristic fluorescence of the liver following the administration of carotene and vitamin A, and also a green fading fluorescence in the pigment layer of the retina, depending on the degree of light adaptation. The recent German investigations of Schairer, Rechenberger, Gockel and Patzelt (14), Patzelt and Schairer (15), and Schairer and Patzelt (16) are accurate and well controlled studies of vitamin A fluorescence. Their observations appeared simultaneously with those of our group in this country. Since the studies were obviously independent they confirmed each other. Their reports and the more extensive ones of our own group represent the basis of the following presentation.

A METHOD OF HISTOLOGICAL DETERMINATION OF VITAMIN A. *1. Physical Principle.* Vitamin A has a maximum absorption in the higher ultra-violet range at 328mμ (17). If exposed to ultra-violet light of similar wave length, vitamin A assumes a bright green, rather quickly fading fluorescence (18, 19). With given solvent and illumination, the fluorescence is the brighter and the fading the slower the higher the concentration of vitamin A. If the illumination is reduced, *e.g.*, by screening out some of the light, the fluorescence is duller, but the fading protracted. Similarly, in certain solvents equal amounts of vitamin A fluoresce more brightly and fade faster than in others (20).

2. Principle of Fluorescence Microscopy. The visible picture is observed which is produced if ultra-violet rays strike fluorescent structures in the examined specimen. These structures change the ultra-violet light which they absorb to visible light of different wave length. The remaining ultra-violet rays are absorbed by a filter in the eye piece (*e.g.*, Leitz ultra-violet protecting filter no. 8574-A).

Various applications of fluorescence microscopy in technical and biological sciences have been reported (21, 22, 12). In biology, either living objects, usually in incidental light, (vital-microscopy, see Ellinger 12, Singer 23) or specimens, usually in transmitted light, have been studied. The fluorescence microscopic picture of human organs has been described in detail (Hamperl 24, Sutro 25, and others). Furthermore, staining with fluorescent dyes (fluorochromy) has been applied (22).

3. Fixing. Fixation in solution of formaldehyde U.S.P. 1:10, up to eight hours in animal organs, up to eighteen hours in human organs, does not affect the vitamin A fluorescence. The sections are mounted in water and examined immediately (14, 26). The common mounting media for frozen sections either yield a disturbing fluorescence (glycerine) or dissolve vitamin A (mineral oil).

4. Microscope. The light of a mercury vapor bulb is filtered through an ultra-violet filter (such as the Corning glass color filter no. 584) to eliminate the visible light. The residue of red rays is absorbed by a glass cell containing

a 5 per cent cupric sulfate solution or an additional Corning glass color filter no. 428. Since the vitamin A fluorescence in tissue sections is produced by rays in the longer ranges of the ultra-violet which are not absorbed by glass, a standard microscope with a standard Abbe condenser is used (27, 15).

5. Observation. Under the fluorescence microscope a dark field is seen in which fluorescent material stands out. Nearly all tissue constituents reveal some kind of fluorescence. This permits orientation in an unstained tissue section. Vitamin A fluorescence is considered any fading fluorescence of green hue in contrast to other types of green fluorescence which are ultra-violet stable.

6. Localization of the Fluorescence. In frozen sections stained with hematoxylin or methylene blue the vitamin A fluorescence is visible (7, 26). If the ultra-violet filter is changed to a ground glass filter the sections can be studied in visible light and the fluorescence localized. For the study of fat distribution, sections were examined after treatment for three minutes with a 0.1 per cent aqueous phosphin 3R solution. By this method fats reveal a silver-white fluorescence on a brown background. This procedure permits more and finer droplets to become visible than is possible with the routine fat methods, because of the water solubility of the dye (27).

7. Photography. Vitamin A fluorescence is photographed by the use of a 35 mm. camera with a periscope view finder on the microscope attachment. The film material should be either of maximal daylight sensitivity or sensitized for green and yellow (28).

Evidence for the specificity of the vitamin A fluorescence. Several points of evidence which are partly suggestive and partly conclusive, support the belief that the green, quickly fading fluorescence in tissue sections is due to the presence of vitamin A.

1. Suggestive Evidence (as to the fluorescence in human and animal organs). A. *Visual appearance of the fluorescence.* The green fading fluorescence of adrenal and liver sections stimulates under the microscope the fluorescence of droplets of an oil-in-water emulsion of oils containing vitamin A, such as percomorph or halibut liver oils (8), various commercially available vitamin A concentrates or solutions in corn oil of vitamin A alcohol, vitamin A acetate or vitamin A palmitate (27). Attempts to supplement the visual observation by spectroscopic analysis of the green fluorescence in tissue sections, by Querner (8), Schairer and his co-workers (14) and ourselves, did not succeed because of the quick fading of the fluorescence. However, if the light which excites the fluorescence, is roughly analyzed by the use of glass filters, the rays producing the green, fading fluorescence in tissues are localized between 310 and 350 mμ, that is, in the neighborhood of 328 mμ, the specific absorption maximum of vitamin A.

Carotene imparts under the microscope a dull, very slowly fading green fluorescence which is visible only in higher concentrations and which is easily differentiated from the vitamin A fluorescence (14, 26).

The biologically inactive anhydro ("cyclized") vitamin A (29, 17) gives chemical reactions similar to those of vitamin A, but imparts not the charae-

teristic green, quickly fading fluorescence of vitamin A, but a dark brown fluorescence which slowly changes to a duller green and then slowly fades.

Corn oil and cotton seed oil do not show vitamin A fluorescence. The following fat soluble biologic substances dissolved in corn oil as tested under the fluorescence microscope in an oil-in-water emulsion do not show a green, quickly fading fluorescence: Several vitamin D preparations, alpha- and mixed tocopherol, synthetic vitamin K (2 methyl-3-phytyl-1-4 napthoquinone), estrone, alpha-estradiol, testosterone, progesterone, desoxycorticosterone, cholesterol, cholesterol esters and 1,2,5,6 dibenzanthracene.

B. *Histo-chemical analysis.* The green, fading fluorescence in the tissue sections is abolished by reagents which either dissolve or destroy vitamin A and is not changed by reagents which have no influence upon vitamin A (7, 14, 26).

C. *The distribution of vitamin A fluorescence.* Its distribution in animal or human organs agrees with that found by chemical analysis using the antimony trichloride (Carr-Price, 30) reaction, by spectroscopic analysis, and by biological assay as far as they are available (31, 32,33, 34).

D. *The relation of the characteristic fluorescence to the vitamin A metabolism in human beings.* In a large series of human livers obtained at biopsy or autopsy, a fairly good agreement has been found between the amount of vitamin A fluorescence and the concentration of vitamin A as determined chemically (35), this partly in contrast to the findings of Schairer *et al.* (14) who considered the existence of a type of vitamin A, which is chemically demonstrable, but not fluorescent in tissues. Experience permits one to predict the result of the chemical analysis on the basis of the histologic picture. In diseases associated with prolonged malnutrition or in other conditions in which the chemical analysis yielded traces of vitamin A, usually very small amounts of vitamin A fluorescence were found (27). After feeding of high doses of vitamin A before operations, very high amounts of vitamin A have been found in the biopsy specimens obtained during the operation (36, 35).

E. *The characteristic fluorescence in deficiencies other than vitamin A deficiency.* In rats deficient in vitamin B_1, B_2 and D, as well as in guinea pigs deficient in vitamin C, a normal distribution of the vitamin A fluorescence was seen (37).

2. Conclusive Evidence as to the Fluorescence in Organs of Rats. A. *Depletion experiments.* While rats are kept on a vitamin A deficient diet, the green, fading fluorescence in liver, adrenal, ovary and lung gradually disappears (14, 15) parallel with a decrease of the liver vitamin A as demonstrated chemically (38, 39). The fatty deposits in the livers of vitamin A deficient rats intoxicated with carbon tetrachloride did not show vitamin A fluorescence: fat appearing in the liver reveals the green, fading fluorescence only if vitamin A is available (40).

B. *Repletion experiments.* If vitamin A is fed to rats depleted of vitamin A, a green, fading fluorescence reappears in typical distribution (14, 15) and the amount of this fluorescence found agrees with the amount of vitamin A fed and with the amount found chemically in the liver (38). If much vitamin A is fed over a prolonged period, parallel with an extreme increase of the vitamin A concentration, a very striking vitamin A fluorescence appears in organs normally

showing moderate fluorescence and some in organs normally free of vitamin A (11, 13, 14, 38, 39). If carotene is fed, after a somewhat longer interval vitamin A fluorescence reappears (15, 38), again parallel to the amounts of carotene given and to the vitamin A content of the organ. Feeding of other substances such as different oils or vitamins failed to produce the characteristic green fluorescence in the organs of vitamin A deficient rats (38).

It was important to rule out the possibility that the green, fading fluorescence in tissue sections is due to riboflavin which, dissolved in the aqueous phase of a water in oil emulsion, shows such a fluorescence. Indeed, Hirt and Wimmer (41, 11) considered a part of the green, fading fluorescence seen in living tissues to be due to vitamin B_2. However, the green, fading fluorescence in fixed tissues is dependent upon the alimentary intake of vitamin A and independent of the intake of B_2, and is not affected by reducing agents (14, 38). Possibly, the water soluble riboflavin disappears from the tissues during fixation and preparation of the sections (38).

Summarizing, conclusive evidence exists that the green, fading fluorescence in sections of rat organs is due to vitamin A. For human organs only suggestive evidence is available. Consequently, in describing the green, fading fluorescence in rats one is justified in calling it vitamin A. In other animals or in human organs, one should speak of vitamin A fluorescence, which indicates that it can be caused by vitamin A but does not exclude other, so far unknown, substances.

Stability of the vitamin A fluorescence. Since vitamin A is quickly destroyed by exposure to air or oxidizing agents (42) the vitamin A fluorescence disappears from frozen sections within a few hours. The speed of disappearance is greater in animal than human organs with variations between different organs. It vanishes much faster from the Kupffer cells than from the epithelial cells of the liver, although the fat distribution remains unchanged. It disappears much faster from tissue sections kept in water than from those kept in plasma, serum, or liver emulsion. This indicates the presence of a protective principle. This principle which in smaller amounts is found in urine, bile and organ emulsions is thermostable, not ether soluble and is more effective at 37° than at room temperature. Its biologic significance is still problematic (43).

Roentgen irradiation does not decrease the vitamin A fluorescence (44), contrary to what Querner (45) reported.

Influence of carrier substances on vitamin A fluorescence. As in non-biological solvents, the vitamin A fluorescence in tissue varies in character, depending on the carrier for vitamin A. In larger fat droplets it is usually less bright, but more slowly fading than in smaller ones. Another factor is the presence of pigments which screen the ultra-violet rays out, thus reducing the brightness, but retarding the fading of the fluorescence. The presence of carotene in the human liver explains why the fluorescence is more slowly fading but duller than carotene-free rat livers. These variations in brightness with equal amounts of vitamin A prevent an exact quantitative determination of vitamin A in tissues by fluorescence microscopy.

Relation Between Fluorescence and Vitamin A Concentration. Vitamin A

fluorescence is visible in droplets of an oil-in-water emulsion if the oil contains more than 20 micrograms of vitamin A per gram, whereas the dull carotene fluorescence is revealed by oils containing more than 150 micrograms per gram. The vitamin A concentration of the individual fluorescent detail in the tissue is above this level. In liver, containing more than 1.5 micrograms of vitamin A per gram, vitamin A fluorescence is encountered (38). In organs in which only certain structures contain vitamin A, the histological method is far superior to the chemical analysis.

VITAMIN A DISTRIBUTION IN DIFFERENT ORGANS UNDER NORMAL AND PATHOLOGICAL CONDITIONS. *Liver.* The liver is the chief depot of the body for vitamin A (31, 32). In the livers of human beings, rats, rabbits, monkeys, dogs, guinea pigs and frogs, vitamin A fluorescence has been described in several sites (14, 26, 38, 46).

I. *In the Kupffer cells.* The fine fat droplets which normally fill the cytoplasm of the Kupffer cells (47) reveal vitamin A fluorescence, except in nutritional deficiency (38). Their absence is uncommon but not necessarily pathologic.

II. *In the epithelial cells.* (a) Fine fat droplets occur near the borders adjacent to the sinusoids. These droplets, demonstrated with sensitive fat stains, are arranged like a string of beads. If large amounts of vitamin A are present those droplets reveal marked fluorescence. They may enlarge and then several of them fill the cytoplasm. (b) Large fat drops, demonstrated by routine fat stains. One of them fills the cytoplasm and usually pushes the nucleus to the side. Their vitamin A fluorescence is very variable and usually lower than that of the small droplets, even in livers containing much vitamin A. (c) Fine bars or droplets, irregularly aggregated throughout the cytoplasm and only sometimes lining up on the edge. Their quickly fading fluorescence is hardly recognized under low magnification; they are not demonstrable by phosphin 3R. Their appearance simulates that of mitochondria (48). That mitochondria contain vitamin A has been proven chemically (49) and suggested by histo-chemical studies (4, 5). Mitochondria of germ cells contain also carotenoid pigments (50). (d) Lipofuscin (wear and tear pigment), which has been considered as a protein compound colored by carotene (51). If the granules show fat reaction and vitamin A fluorescence their red-brown fluorescence appears only after the latter has faded. (e) The cytoplasm imparts a diffuse green, fading fluorescence which is removed by alcohol. It seems to be due to small amounts of vitamin A diffusely spread over the cytoplasm.

The different sites of vitamin A fluorescence in the liver cause a great variability of the picture with and without relation to the lobular topography. The significance of this polymorphic distribution is as yet unexplained and only a few points can be gathered from experiments on animals under controlled conditions.

I. *Influence of Various Experimental Conditions Upon the Vitamin A Distribution in the Rat Liver.* A. *Influence of diet.* 1. *Hypervitaminosis A.* Livers of rats, which had received large doses of vitamin A for several weeks and showed the signs of hypervitaminosis A (52, 53, 54, 55), reveal an extremely strong

vitamin A fluorescence. The enlarged Kupffer cells are very strongly fluorescent and rich in fat (54, 56), whereas the epithelial cells show the usual fluorescence, chiefly imparted by fine fat droplets and also by the cytoplasm. Around the periportal and central veins, fine fluorescent fat droplets appear in endothelial cells and other connective tissue cells (38, 39).

II. *Unbalanced Nutrition.* Rats kept for more than three weeks on otherwise balanced diets consisting predominantly of protein, carbohydrate or fat respectively (see 57), reveal characteristic variations partly paralleling that of the fat distribution (58). The rats on a high protein diet show less vitamin A than the others, much of it localized in Kupffer cells; in the lobular centers many mitochondria are seen in the epithelial cells, whereas in the periphery fine droplets line their edge. In the animals fed much fat, most of the vitamin A is localized in medium-sized fat droplets on the edge of the liver cells, less in Kupffer cells and some in isolated large fat droplets on the periphery. In those given much carbohydrate the most vitamin A, but also the most morphologically demonstrable fat (59), was seen; the strongly fluorescent fat droplets near the border of the cells increased in size towards the periphery of the lobule.

In starving rats, the chemical vitamin A concentration does not significantly change (60) despite marked microscopic alterations in fluorescence. The vitamin A fluorescence becomes irregular, and moderately fluorescent large fat droplets accumulate at the lobular periphery. When in later stages no fat is morphologically demonstrable, the cytoplasm imparts a strong green, fading fluorescence indicating that the latter carries most of the vitamin A instead of the visible fats (58).

III. *Choline Deficiency.* In rats on choline deficient diets (supplemented by carotene), in which fatty livers develop (61), the vitamin A fluorescence of the fat droplets in the liver markedly decreases. First, irregular foci remain in which Kupffer cells and fat droplets in the surrounding liver cells impart the fluorescence; later, only a few isolated fat droplets reveal some vitamin A (62). If vitamin A instead of carotene is fed, the Kupffer cells maintain vitamin A, whereas the liver cell fat is devoid of it. It appears that choline or related substances (labile methyl groups) are connected with the presence of vitamin A in the fat of the liver cells. If additional studies corroborate the impression that in choline deficiency vitamin A storage in the Kupffer cells occurs only after vitamin A and not after carotene feeding, it would indicate that only the liver cells are able to split vitamin A into carotene, something they are unable to do in a fatty liver. It has been repeatedly claimed that liver damage interferes with the conversion of carotene into vitamin A (63, 64, 65, 66 and others).

B. *Experimental Liver Damage.* I. *Intoxication with Hepato-Toxic Drugs.* Intoxication with drugs with possible hepato-toxic effect, as phosphorus, bile acids, thyroxin, phenolphthalein and sulfanilamide, causes a shift of the vitamin A fluorescence in the liver (40) without necessarily reducing the hepatic vitamin A concentration (63, 67, 68, 69, 70). Within one day after administration of carbon tetrachloride (40) the fluorescent small fat droplets near the border of the liver cells enlarge in the lobular center which leads to an accumulation of

strongly fluorescent medium-sized droplets. Following repeated administration, the vitamin A disappears from the Kupffer and liver cells in the uninvolved peripheral area and is seen only in the central fatty areas. After more than one month, only around the central area a few large fat droplets and some histiocytes reveal vitamin A fluorescence, whereas fatty areas in the intermediary and even peripheral zone are no longer fluorescent; the chemical concentration in this stage is reduced (see also Haig and Post, 71). If the rats receive high vitamin A supplements, the vitamin A fluorescence in the fatty area is more marked and the Kupffer and liver cells in the uninvolved area retain it longer. With a high carbohydrate diet the fatty changes are less marked than with high fat or protein diet and the normal Kupffer and liver cells keep vitamin A longer. During recovery from this intoxication, rats on a high protein diet show vitamin A in the Kupffer and liver cells of the uninvolved areas before rats fed much fat or carbohydrate do (faster regenerations).

II. *Cirrhosis.* In animals with experimental dietary cirrhosis as produced by György and Goldblatt (72), the vitamin A distribution is the more irregular the farther advanced the liver damage. In early stages a non-lipid soluble material with non-fading, gold-brown fluorescence is seen in liver cells and the adjacent Kupffer cells. Later it accumulates in connective tissue cells of the proliferated periportal spaces (73). This material, which is fluorescent even in paraffin sections, is apparently a break down product of fats. It seems identical with ceroid, described by Goldblatt and György (72) and Lillie et al. (74).

III. *Carcinogens.* Some carcinogenic hydrocarbons reduce the vitamin A stores in the liver (75, 76, 77, 78), especially that of the mitochondria (49). However, in the recovery a normal fluorescence microscopic picture is seen (Marron, 79).

C. *Depletion of Vitamin A Depots.* Rats put on a vitamin A deficient diet immediately after weaning lose their small vitamin A stores within 22 days, before their weight becomes stationary or clinical signs of vitamin A deficiency appear (15, 38). The fluorescence of the cytoplasm disappears first, then that of the fine droplets near the border of the liver cells, and finally that of the Kupffer cells; fat droplets retain it the longest. If rats are put on the vitamin A deficient diet a few days after weaning their depletion is retarded (see 80). Adult stock animals need about six months for depletion. Albino rats are more quickly depleted than pigmented ones (16).

If rats are first made hypervitaminotic and then put on a vitamin A deficient diet (39) fluorescence of the Kupffer cells decreases first; that of the liver cells remains. The marked preponderance of the fluorescence of the Kupffer cells gradually subsides, after the vitamin A concentration drops to a third of that in the hypervitaminotic stage. The decrease of the Kupffer and liver cell fluorescence is then parallel. In advanced hypovitaminosis droplets are found in the epithelial cells very close to the Kupffer cells and once more a preponderance in the Kupffer cells appears. Finally, a few Kupffer cells show the last remnants of fluorescence. Male rats are depleted faster than female rats. The serum vitamin A, which was higher in males than in females, was not related to the fluorescence. These experiments indicate that the Kupffer cells not only

distribute vitamin A but also destroy it. They apparently cause the rapid vitamin A loss in hypervitaminotic animals on a vitamin A deficient diet (81). Whether this non-economic utilization is related to a deficiency in vitamin E (82) remains to be examined.

D. *Repletion of Vitamin A Depot.* After feeding of more than 250 units of vitamin A to rats, the liver of which is free of vitamin A for a few days, the fluorescence reappears (15), first in the Kupffer cells and then in small droplets in the adjacent part of the liver cells. The speed of reappearance depends on the amount fed (38). In animals depleted for more than a week the repletion is irregular, apparently due to disturbance of intestinal absorption. The results of feeding carotene are variable and the fluorescence appears later and in smaller amount; before the appearance of vitamin A fluorescence, the epithelial cells of the liver reveal a diffuse, faint, green, slowly fading fluorescence, probably due to the temporary presence of carotene (15, 38) For replacement of vitamin A by subcutaneous or intraperitoneal administration, larger doses and a longer interval are required (see 83, 84, and others). Carotene administered parenterally did not restore the vitamin A fluorescence (15, 38). After feeding of vitamin or carotene to rats, intoxicated by carbon tetrachloride, the fluorescence appears in the large fat droplets of the liver and only with large doses in the unaltered areas. This indicates a higher avidity of the fatty areas for vitamin A (40).

2. Variations in the Distribution of Vitamin A Fluorescence in the Human Liver. The marked variations encountered were not caused by postmortal changes since specimens of livers obtained for biopsy during laparotomy reveal similar pictures (36). Certain findings were characteristic even in respect to the marked polymorphism (26, 85, 35, 86).

A. *Fat Deposition.* Localized or generalized infiltrations of medium-sized and large fat droplets may show absence of, moderate, or strong, vitamin A fluorescence. In alcoholics, the picture often resembles that of the choline-deficient fat liver (62). This raises the question whether similar dietary factors may not decide the vitamin A fluorescence of the large fat droplets in the human liver.

B. *Preponderance in Kupffer Cells.* In the presence of huge amounts of vitamin A, *e. g.*, after feeding of large doses, most of the fluorescence is in the Kupffer cells and the liver cells have a normal appearance. However, in some conditions, like diabetes mellitus, uremia, and obstructive jaundice, the Kupffer cells reveal strong fluorescence, whereas the liver cells are almost devoid of it. In visible light these areas show usually a toxic edema. Since in diabetes clinical signs of vitamin A deficiency occur (87), possibly the lack of vitamin A in the liver cells with accumulation in the Kupffer cells interferes with its utilization.

C. *Focal Parenchymal Damage.* The vitamin A fluorescence may either disappear from the area involved as in recent thrombosis of the portal vein or with necrosis of the lobular center, or fluorescent material may accumulate in peculiarly shaped sites as in irregular fat droplets in the liver cells or in bizarre shaped Kupffer cells.

D. *Hepatitis.* The greatest polymorphism occurs in acute hepatitis. In

acute yellow atrophy very much vitamin A fluorescence may be imparted by irregular shaped fat droplets in liver cells; all visible fat is fluorescent. In subacute atrophy less fluorescence is seen and some livers are almost devoid of it. In atrophy with transition into cirrhosis, histiocytes and endothelial cells in the vascular connective tissue may be rich in vitamin A fluorescence (see chemical study of Cox 88).

E. *Liver Cirrhosis.* In arrested forms, the lobules and regenerated pseudolobules reveal a nearly normal distribution. Fatty areas may show much or little vitamin A fluorescence. Connective tissue cells in proliferated periportal fields often contain fine fat droplets with fluorescence. In progressive cirrhosis, especially with jaundice, only scattered droplets in liver cells or some bizarre shaped Kupffer cells reveal vitamin A fluorescence. The decreased fluorescence, often despite high fat content, parallels the low chemical vitamin A concentration (89, 90, 91, 92, 64).

F. *Irregular Distribution.* In addition to the described alterations others occur which are less easily classified. The fluorescence pattern may vary from cell to cell or from cell group to cell group without difference of the histological picture in visible light. The irregularity of the vitamin A distribution is not mirrored by the chemical analysis. Marked degrees of disturbance of the vitamin A fluorescent pattern are associated with parenchymatous damage upon routine histological analysis or clinical study of the liver function (85). Consequently, milder degrees of this disturbance are considered as initial liver damage not recognized by the common routine histological methods (35, 36). According to this standard and in comparison with normal experimental animals almost no human liver available for examination is entirely normal.

G. *Age Influence.* In the fetus much vitamin A fluorescence is found in liver and Kupffer cells. Toward term it markedly decreases and in new born infants only traces are seen. In the second month, an increase starts and after the second year the vitamin A distribution approaches that of the adult (26). These findings agree with chemical analyses (93, 94, 95, 96).

H. *Estimation of the Total Amount of Vitamin A Fluorescence.* The total amount of fluorescence was low, in primary liver disease, in obstructive jaundice, malnutrition resulting from tumor and also in renal conditions; this agrees with chemical analyses (92, 95, 97 and others).

3. Significance of the Vitamin A Distribution in the Human Liver. A few points can be made so far from the study of vitamin A fluorescence.

A. *The Rôle of the Kupffer Cells.* The rôle of the Kupffer cells in fat (98) and vitamin metabolism (11), especially in that of vitamin A (99), has been stressed. The Kupffer cells are the last to be depleted and the first to be repleted. They absorb vitamin A from the blood and transmit it to the liver cells, as seen from its presence in the portions of the liver cells adjacent to the Kupffer cells; a block in this transmission is probably responsible for the picture in toxic edema. They contain the excess of vitamin A in hypervitaminosis and probably destroy it (46). They store carotene if it is injected in aqueous solution (66). After blockade of the Kupffer cells the storage of vitamin A in the liver is interfered with (100, 68, 101).

B. *The Rôle of the Liver Cells.* The liver cells are the permanent depots for vitamin A. In liver damage they may be unable to hold it or release it to the blood. From large fat droplets vitamin A is very slowly released (46).

C. *Relation Between Plasma Vitamin A Concentration and Distribution, and Amount of Vitamin A Fluorescence* (35). Comparison of the plasma vitamin A level with hepatic vitamin A concentration and fluorescence, in specimens of liver removed for biopsy, over a wide range of the plasma vitamin A level shows a parallelism only if an average of a number of cases is taken. In individual cases no definite relation between plasma level and liver stores exists. High plasma level usually indicates normal or high liver stores, whereas low levels may be associated with high or low liver concentration. The plasma level runs somewhat parallel to the vitamin A fluorescence of the Kupffer cells, less so to that of the epithelial cells. With marked discrepancy between plasma level and hepatic vitamin A concentration the vitamin A pattern is very much disturbed, and liver function is impaired.

D. *Vitamin A Shift in the Liver as a Cause of Functional Avitaminosis.* The shift of the vitamin A from normal to pathologic sites in the liver may explain the drop of the plasma vitamin A level found in acute liver diseases or in pneumonia. (Literature, see 102.) A lack of vitamin A intake cannot explain this phenomenon since months are required to reduce the plasma vitamin A level of healthy adults by a vitamin A deficient diet (103, 104, 105). Apparently, the liver regulates the plasma vitamin A level and that explains the parallelism between liver stores and blood level in healthy animals (106, 107, 108). In liver damage in which vitamin A has shifted to places where it is not available for immediate release, the plasma vitamin A level may drop in the presence of quantitatively sufficient liver stores and a functional avitaminosis exist due to disturbed release (109, 97, 35). In healthy young rats clinical signs of vitamin A deficiency appear only a long time after vitamin A has been discarded from the liver, whereas in human beings the liver is never completely free of vitamin A even if clinical signs of vitamin A deficiency appear, such as hemeralopia as seen in liver diseases (110, 111, and others).

The value of vitamin A therapy in those cases is apparent from the histologic picture (112). Large doses have to be given in order first to saturate the pathologic sites which have an increased avidity for vitamin A and then to enforce storage in the normal sites from which vitamin A is more readily available for use. Actually, giving large doses to carbon tetrachloride intoxicated rats (40), or human beings with liver damage (38) resulted in storage of vitamin A in normal sites.

4. Fish Liver. In fish livers (113) fluorescence is imparted by medium sized fat droplets which fill the cytoplasm of the cells, the Kupffer cells being not fluorescent. In salt water fish, the green fluorescence is of extreme strength and the liver structure can be made out only after the fluorescence has partly faded. In fat stains of livers from fresh water fish an identical picture was found. However, only a few of the fat droplets imparted the green, quickly fading fluorescence, whereas the majority show a yellow-brown, slowly fading fluorescence. This is due to vitamin A_2, a factor with an absorption spectrum

at 352 mμ, demonstrated by physico-chemical analysis in livers of fresh water fish in addition to small amounts of vitamin A_1 (114, 115, 116). Oils extracted from fresh water fish showed the yellow-brown fluorescence. If fed to vitamin A deficient rats, the yellow-brown fluorescence appears in the liver distributed in a manner similar to that of the usual green fluorescence and, as described (118 116, 117), growth of the rats continues. The different histologic distribution of vitamin A in the livers of fish supports the idea that vitamin A plays a special rôle in the metabolism of fish (33).

Adrenal gland. In the cortex of the adrenal of the rat large amounts of vitamin A are present in small lipoid droplets in the epithelial cells of the fascicular layer. The glomerular layer, despite identical fat distribution, is free of vitamin A, even in hypervitaminosis in which the fluorescence extends almost to the medulla. In rats with low vitamin A reserves, the outer zone of the fascicular layer retains it (38).

In the human (26), vitamin A fluorescence is found in the fascicular layer of the cortex and to a minor degree in the glomerular layer, also in the central cortex close to the medulla. The fluorescence is less vivid and fades more slowly than that of the liver. Double refraction and vitamin A fluorescence were often concomitant and at times in identical distribution. The reticular layer reveals brown-red fluorescent particles (lipofuscin) which are also seen in the fascicular layer after fading of the vitamin A fluorescence. The variations of the vitamin A fluorescence are not necessarily parellel to that of the lipid content. Only small amounts or traces are observed in malnutrition and diseases of the liver, also in infectious diseases, here probably related to the depletion of the lipids (119, 120). The extensive changes of the human adrenal in early postnatal life (121, 122) influence the vitamin A distribution. During the entire first year only small amounts are found and then the storage gradually approaches that in the adult.

The adrenals are a storage place for various lipoids and one of the substances in the lipid mixture gives vitamin A fluorescence. Its slow fading may be caused by the presence of carotenoids which are responsible for the yellow color of the organ.

Testicles. In human testicles (26) after puberty, different fluorescent details appear which partly reveal a green, fading fluorescence suggestive of vitamin A: brown pigment in the cells of Leydig, and lipoid droplets and lipofuscin in the Sertoli and spermatogenic cells. The fluorescence of the Leydig cells is strong but slowly fading. The vitamin A fluorescence seems related to the activity of the testicle, appearing with maturity and disappearing with involution. In some animals the interstitial cells of Leydig show strong vitamin A fluorescence, whereas in others it is missing.

Ovary. In the rat ovary (38) a dull but slowly fading vitamin A fluorescence is seen in the interstitial cell cords and a bright and rapidly fading one in the so-called corpus luteum. In vitamin A deficient rats no vitamin A is seen in the ovary; upon feeding, it reappears.

In the human ovary the vitamin A fluorescence changes during development

and with the menstrual cycle, as studied on material obtained at autopsy (26) and from surgical operations upon women whose menstrual history could be ascertained (123). Three types of characteristic fluorescence are encountered: 1, a very dull, very slowly fading, diffuse, green fluorescence in the cytoplasm of the granulosa cells of the corpus luteum, probably due to carotene (carotene is found chemically in the ovary (124) and causes the yellow color of the corpus luteum); 2, a bright quickly fading fluorescence seen particularly in fine fat droplets in granulosa cells, and 3, a little duller, more slowly fading one in fat droplets in theca cells.

Vitamin A fluorescence appears around the third month of postnatal life, in fine fat droplets in the stroma, and in granulosa and theca cells of the Graafian and atretic follicles. In the reproductive stage the granulosa cells of the maturing follicle reveal fine fluorescent droplets. They increase in size and number after ovulation as the corpus luteum forms. The appearance of the granulosa cells in the mature corpus luteum suggests the possibility that the fluorescent droplets are secreted into the capillaries. With involution the picture of secretion changes into that of retention. The shrinking granulosa layer contains large, strongly fluorescent droplets and fine fluorescent droplets appear in the surrounding tissue. Finally, the vitamin A fluorescence disappears. The corpus luteum of pregnancy shows only carotene and no vitamin A fluorescence. The corpora atretica, which are rich in estrogen and progesterone (125), show strong vitamin A fluorescence of the double refractile lipids in the theca cells. In the course of the atresia it gradually disappears, while a red-brown ultraviolet stable fluorescence persists. The vitamin A fluorescence of the corpora atretica depends on their size and age and the menstrual cycle. The smaller and older the corpus atreticum, the lower the fluorescence. After the climacterium no vitamin A fluorescence is seen in the ovary.

The significance of the morphologic lipid distribution in the ovary and its relation to endocrine function is not established (126, 127, 128). The vitamin A fluorescence, however, seems to be a morphologic sign of endocrine activity. Whether it indicates hormone storage or formation is not clear. As long as the relation of vitamin A to sex hormone activity and even to reproduction is as unclear as at present (literature, see 26, 123), the presence of vitamin A fluorescence at the morphologic sites of sex hormone activity is best explained by solubility of sex hormones and vitamin A in the same lipid carriers.

Uterus. The endometrial lipids, present in certain stages (129, 130), occasionally reveal quickly fading vitamin A fluorescence without parallelism to that in the ovary (123).

Breast. The non-lactating mammary gland is free of vitamin A fluorescence. The fat droplets in the epithelial cells of the lactating gland reveal high amounts of it (14, 26) in agreement with the high vitamin A content of the colostrum (131).

Fat tissue. The fat cells in adipose tissue or scattered in the stroma of various organs impart a dim but slowly fading vitamin A fluorescence, in agreement with their function as vitamin A depots. The lipidic pigments retard the fading

and dim the fluorescence. The different depots of the same individual vary in their fluorescence and show some relation to the nutritional condition (26).

Eye. The presence of vitamin A in the eye is established (see 132). The vitamin A fluorescence, as reported by Querner (8) and Evans and Singer (133), changes depending on the function as described by Jancsó and Jancsó (13), Greenberg and Popper (134), and most carefully by Schairer and Patzelt (16). The pigment coat shows fine fat droplets arranged on the periphery of the epithelial cells. They reveal yellow, ultra-violet, stable fluorescence which may be covered by a green, fading vitamin A fluorescence. In the rod and cone layer, some diffuse vitamin A fluorescence is found in addition to a brown-red ultraviolet stable fluorescence which, according to Schairer and Patzelt (21), is due to visual purple. Vitamin A fluorescence is also imparted by connective tissue cells in the ciliary processes.

The nutritional status determines the vitamin A fluorescence of the ciliary processes just as it does that of other organs, but hardly influences that of the pigment layer. In the latter it is not increased in hypervitaminosis and not apparently decreased in hypovitaminosis. Even in far advanced vitamin A deficiency, long after the other organs have lost their vitamin A fluorescence, it is still observed in the retina and can be found even in the ulcerated eyes from extreme vitamin A deficiency. The state of light adaptation, however, which does not change the vitamin A in the ciliary processes, influences the fluorescence of the pigment layer and rod and cone layer. In dark-adapted animals no vitamin A fluorescence is seen, but the visual purple fluorescence of the rod and cone layer is strong. Upon light adaptation the latter disappears, whereas the vitamin A fluorescence is strong. The appearance of vitamin A upon exposure to light is rapid and takes place also in dead animals; its disappearance in the dark is slow. The fluorescence microscopic examination corroborates the hypothesis of Wald (135), that light changes the visual purple over a transitional stage of visual yellow and the carotenoid retinine to vitamin A and that during dark adaptation the visual purple is slowly rebuilt from vitamin A.

Intestine. The gastro-intestinal tract of the rat reveals vitamin A in its fat cells. Only after feeding of more than 900 units of vitamin does it appear in the wall of the duodenum and small intestine as a sign of its absorption. It is first seen in epithelial cells and in histiocytes and lacteals of the lamina propria of the villi, later on in larger lacteals up to the subserous layer (38). This picture of lymphatic absorption agrees with experiences in the human (136). Recent experiments (137) showed that vitamin A absorption after administration of oily concentrates starts in the upper duodenum, has its peak in the upper jejunum and subsides in the ileum. That does parallel the fat absorption Whereas fat is found in the lumen throughout the entire intestine, its vitamin A fluorescence disappears above the level of the intestine at which the vitamin A absorption ceases, even after feeding of more than 300,000 units. The absorption subsides, therefore, either because vitamin A has been destroyed within the lumen of the intestine or because the entire vitamin A has been absorbed. In the human intestine no vitamin A fluorescence is encountered (26).

KIDNEY. In the kidney three types of distribution of vitamin A fluorescence occur in different animals. In the rat (38) a bright, quickly fading fluorescence is imparted by the endothelial cells of the intertubular capillaries of the cortex. It is marked in hypervitaminotic animals but also in advanced hypovitaminosis or in choline deficiency (62) when in both instances the liver is nearly depleted. Similarly the renal vitamin A concentration increases in depletion of the liver after dibenzanthracene administration (76).

In the normal dog kidney vitamin A fluorescence is imparted by medium sized isotropic fat droplets in the Henle loops.

In the human kidney (26) vitamin A fluorescence is only encountered in renal damage. The fluorescence is seen chiefly in the proximal convoluted tubules in fine droplets at the base of the epithelial cells. In the vicinity, the stromal histiocytes are rich in fluorescence. The vitamin A fluorescence is usually associated with marked fatty changes, but not necessarily with deposition of doubly refractile lipids.

The significance of the different forms of vitamin A fluorescence is unknown. In the human its appearance seems to be associated with the development of the nephrotic syndrome. Vitamin A appears in the urine together with other lipoids in renal diseases, but also in infections (see 138, 139). Together with other lipids and with protein, it seems to be reabsorbed in the tubules and carried from there to the tubular capillaries, sometimes being deposited in the interstitium (140). This explanation would agree with the recent theory on the histogenesis of lipoid nephrosis as a primary disease of the glomeruli with secondary tubular reabsorption of the lipoids and proteins (141, 142).

SKIN. Although the epidermis shows very early manifestations of vitamin A deficiency, no vitamin A fluorescence is encountered in the epithelial layers, even after feeding of high doses of vitamin A. Only the fat cells in the cutis and subcutis reveal small amounts (143).

OTHER ORGANS. In the rat (11, 38), after feeding high doses vitamin A may appear in the endothelial cells and connective tissue cells of lung, serous membrane, meninges and in other places. In the human, fluorescent fat droplets may be seen in the anterior lobe of the pituitary gland (8, 14).

TUMORS. Vitamin A fluorescence is seen in tumors originating from a mother tissue with vitamin A fluorescence, its distribution resembling that of the mother tissues (144). It may be found in hepato-cellular carcinoma, in adrenal cortical adenoma resembling the glomerular layer, in lipoma and in testicular dysgerminoma. Among the ovarian tumors it is chiefly present in those with endocrine activity. It occurs in the masculinizing arrhenoblastoma and in the feminizing group: granulosa cell tumor (in a distribution similar to that of the normal granulosa), theca cell tumor (resembling theca cells) and xantho-fibroma (simulating ovarian stroma with vitamin A fluorescence); while the ordinary fibroma of the ovary is without fluorescence. It was also observed in ovarian dysgerminoma (in a distribution similar to that of the testicle). The picture of a tubular adenoma of the breast resembled the lactating breast. Except for a few examples (*e. g.*, plant cells), the other tumors were free of vitamin A fluorescence.

even if they showed marked fatty degeneration. The presence and distribution of vitamin A fluorescence may help in determing the origin of a tumor. Thus the hypernephroid carcinoma of the kidney often reveals vitamin A fluorescence similar to that seen in the adrenal. This supports the original hypothesis of Grawitz, that those tumors originate from the adrenal (literature, see Ewing 145). Other renal tumors like carcinoma of the pelvis or benign tubular adenoma have no vitamin A fluorescence despite the presence of anisotropic lipids in the latter. That only a few well defined groups of tumors yield vitamin A fluorescence indicates that the local presence of vitamin A does not inhibit tumor growth, neither is it necessary for tumor formation. This is important in view of the repeatedly (see 144, 146, 147) discussed relationship between vitamin A and tumor formation.

Relation of vitamin A fluorescence to fat. The vitamin A fluorescence is, with few exeptions, bound to lipids demonstrable by sensitive strains. Vitamin A (or the other substances yielding the fluorescence) can be considered as exhibiting a fluorescent vital stain characterizing certain lipids of the body. However, since only some lipids reveal vitamin A fluorescence, the presence of vitamin A is not due to a non-specific solubility of vitamin A in the available fats but to a specific affinity of certain lipids for vitamin A (14, 148).

There is an apparent influence of vitamin A upon the fat metabolism in rats put on a vitamin A deficient diet. In agreement with chemical analyses (149), the morphological demonstrable fat disappears from the liver shortly after vitamin A vanishes; after feeding a fatty diet containing vitamin A, fat reappears one or two hours before vitamin A becomes visible (38). Various investigations (78, 76) indicate that vitamin A may occur also in a protein bound form; this is not excluded by the fluorescence microscopic picture.

The relation between the vitamin A depots in the body. In rats on vitamin A deficient diet the fluorescence disappears from different sites in the following order: interstitium of the various organs, adrenal, liver, and kidney; while the retina does not give it up at all. In repletion by oral route it reappears first in the intestine, then in the liver and almost simultaneously in the adrenal, finally in the kidney and the rest of the organs (38).

Upon carotene feeding, sometimes the vitamin A fluorescence appears first in the lamina propria of the villi of the small intestine but usually first in the Kupffer cells and shortly afterwards in fine droplets in the epithelial cells of the liver and in the adrenal (38). The question as to where carotene is split to vitamin A is therefore not yet satisfactorily answered. Most of the evidence points to the Kupffer cells of the liver (see also 100). However, sometimes cells of the intestinal villi or epithelial liver cells seem to split it.

Review of the vitamin A distribution in tissues. So far, not much can be concluded from the histological picture as to the rôle of vitamin A. Where the first morphologic signs of vitamin deficiency appear, namely, the epithelium (55), vitamin A fluorescence is not demonstrable. Actual presence of vitamin A is required for its rôle in vision; it participates in a known chemical process in the retina from which it does not disappear even in extreme deficiency. The

different localizations of vitamin A may be due to the following factors: 1, participation in a chemical process (retina); 2, distribution and possible destruction (Kupffer cells); 3, presence at the site of sterone hormone formation (adrenal, testicle and ovary), probably due to solubility in the lipid carriers of the hormones; 4, storage (fat cells and cells of the interstitial tissue in the rat); 5, absorption (intestine); 6, secretion (lactating breast); 7, pathologic permeability (kidney); 8, imitation of the mother tissue (tumors). Its presence in the epithelial cells of the liver is due to several of the above factors.

CONCLUSION

The microscopic visualization of vitamin A by fluorescence permits the study of its distribution in organs. In various physiologic and pathologic conditions the distribution undergoes characteristic changes, not indicated by chemical analysis or bio-assay. Conclusions as to the rôle of vitamin A in the body can be made which are not arrived at by other methods. The histologic method, which permits only a rough but quick estimation as to the quantity of vitamin A present, does not substitute for the chemical analysis but supplements it. The fluorescence microscopic demonstration of vitamin A, however, not only aids in the investigation of the vitamin A metabolism but also presents a new histologic method for the differentiation of lipids independent of the biologic significance of the fluorescence.

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